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Part II

Department of Health and Human Services

Food and Drug Administration

Bacteriological Analytical Manual, Chapter 29—Listeria Isolation; Revised Method of Analysis; Notice

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

[Docket No. 88D-0367]

Bacteriological Analytical Manual, Chapter 29—Listeria Isolation; Revised Method of Analysis

AGENCY: Food and Drug Administration.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) is providing notice of revised methodology for detecting and confirming the presence of *Listeria monocytogenes* in foods. The revised method will reduce the time required to analyze foods for *L. monocytiogenes* and, therefore, will allow FDA to more quickly obtain analytical results necessary to support the agency's regulatory or administrative actions. The revised method will be incorporated into and replace the *Listeria* isolation method in Chapter 29—"*Listeria* Isolation" of the *Bacteriological Analytical Manual* (6th Edition, Supplement, 1987). The revised *Listeria* method is as analytical sensitive as the method that is being replaced.

FOR FURTHER INFORMATION CONTACT: George J. Jackson, Center for Food Safety and Applied Nutrition (HFF-234), Food and Drug Administration, 200 C Street, SW., Washington, DC 20204, 202-245-1051.

SUPPLEMENTARY INFORMATION: The analytical methods used by FDA for the microbiological examination of foods and cosmetics are published by the Association of Analytical Chemists (AOAC) in a manual entitled *Bacteriological Analytical Manual* (BAM). Chapters contained in the BAM each generally deal with the methodology that is to be utilized for the isolation of particular microorganisms, are subjected to a peer review process by scientists within FDA, as well as scientists not affiliated with FDA, and must be determined to be scientifically justified and acceptable before they are published in the BAM.

The revised *Listeria* isolation method has undergone the peer review process and has been determined to be scientifically justified and acceptable for publication in Chapter 29 of the BAM. The revised method is as sensitive as the method that is being replaced. In addition the revised method will save analytical time and will allow FDA to more quickly determine analytical results necessary to support regulatory or administrative actions involving *L. monocytogenes*.

Before these changes, all FDA regulatory actions taken since 1985 against food adulterated with *L. monocytogenes* have been based on the method widely known as the "Lovett Method" (BAM, 6th Edition, Supplement, 1987). This method requires that portions be taken from enrichment broth cultures at 24 hours and 7 days and streaked onto the surface of the selective modified McBride agar. Full confirmation of *L. monocytogenes* requires additional tests on bacteria isolated from the selective medium. A total of 10 to 11 days is needed to isolate and identify *L. monocytogenes* from a food sample.

Many new methods have been developed since 1985 and reported in the scientific literature, some specific for *L. monocytogenes*, others for detection of the genus *Listeria*. Detection methods for a pathogenic bacterium historically undergo periodic modifications as more is learned about the bacterium, its ecology and characteristics, and the applicability of a method for determining presence of the bacterium in a specific food. Thus, the evolution of methodology for *L. monocytogenes*, as for other microganisms, is a reflection of normal scientific progress.

FDA has found that the following changes of the *L. monocytogenes* testing procedures will reduce the total time it takes to confirm the presence of *L. monocytogenes* in a food from 11 days to 5 to 6 days:

1. Isolation from the enrichment culture by streaking onto the surface of lithium chloride-phenylethanol-moxalactam (LPM) plating mediun in addition to the usual modified McBride agar medium.

2. Elimination of the alkali (KOH) pretreatment before streaking onto modified McBride agar. This pretreatment will also not be done in conjunction with the LPM medium.

3. Reduction of the enrichment culture incubation time from 7 days to 2 days. Hence, the enrichment culture will be sampled at both 1 and 2 days only.

. Data show that no sensitivity is lost by reducing the 7-day enrichment to 2 days if the LPM plating medium is used in addition to the currently used modified McBride agar medium. Comparative studies have been conducted by FDA in which a variety of foods were "spiked" with known numbers of healthy and heat-injured *L. monocytogenes* over a broad range of decimal dilutions. Comparisons of recovery between the 7-day enrichment plated onto McBride agar (as currently done by FDA) and a 2-day enrichment plated onto both McBride and LPM agars show essentially no differences in recovery efficiency.

In addition, FDA has made the following minor modifications:

1. Provision for the optional use of a concave mirror for the Henry oblique illumination system according to the preference of the observer; the obliquely illuminated colony may be viewed unaided or with low power microscopy. The discretional use of positive and negative control colonies to "tune" the observer's eyes is recommended.

2. Inclusion of references to rapid methods in the BAM, but these methods are not described in detail. They are mentioned solely as optional supplemental methods that give advance warning or identification of pathogenic and/or nonpathogenic *Listeria*. They are not intended in any way to be substituted for any part of the BAM *Listeria* isolation procedure which is FDA's current, definitive test.

FDA notes that it is not obligated to publish in the **Federal Register** changes in methodology contained within the BAM (21 CFR 10.90(b)(10)). However, because of the public health ramifications associated with *L. monocytogenes*, FDA believes that it is the public interest to provide notice in the **Federal Register** of the revised methodology for isolating *L. monocytogenes* and is therefore publishing this revised method.

Dated: October 26, 1988.

John M. Taylor,

Associate Commissioner for Regulatory Affairs.

FDA BACTERIOLOGICAL ANALYTICAL MANUAL

Chapter 29. *Listeria* Isolation

(Revised 13 October 1988)

Joseph Lovett and A.D. Hitchins

The ninth edition of *Bergey's Manual of Determinative Bacteriology* (1) lists eight species in the genus *Listeria: L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, L. grayi, L. murrayi,* and *L. denitrificans*. Recently, *L. dentrificans* has been allotted to a new genus, *Jonesia* (2). Differentiation of the seven accepted species is possible by Table 1. Stuart and Welshimer (3) have proposed a new family, *Listeriaceae*, to contain two monospecific genera, *Listeria* and *Murraya*. Under this classification system, *L. monocytogenes* would contain a heterogeneous group of organisms distinguishable from *Murraya* spp. by their inability to ferment mannitol. The genus *Murraya* would

contain two subspecies, *M. grayi* ssp. *grayi* and *M. grayi* ssp. *murrayi*, distinguishable by the ability of *M. grayi* ssp. *murrayi* to reduce nitrates. In this chapter we list the two species as they appear in the ninth edition of Bergey's Manual (1). Two groups within *L. monocytogenes*, the hemolytic pathogenic strains and the nonhemolytic nonpathogenic strains, were readily apparent. Nonpathogenic *L. monocytogenes* (4) became known as *L. innocua* from the Latin word harmless. Two other nonpathogenic species, *L. welshimeri* and *L. seeligeri* (5), are now recognized.

The most recently described species is *L. ivanovii* (6), a strongly hemolytic genomic group formerly called *L. monocytogenes* serovar 5 and *L. bulgarica*. It is associated with animal pathology and is rarely isolated from human sources. Only *L. monocytogenes* is consistently associated with human illness. Both *L. ivanovii* and *L. monocytogenes* are pathogenic for mice.

The classical tests for *Listeria* pathogenicity are the Anton test (rabbits), inoculation of mice, and inoculation of embryonated eggs (7). The mouse test, using intraperitoneal (i.p.) injection is most frequently used and is described later in this chapter. Recently, the immunocompromised mouse model of Stelma *et al.* (8) has become available. It is recommended because of its greatly improved sensitivity. Animal confirmation of *L. monocytogenes* pathogenicity is not routine for clinical isolates and is optional for regulatory isolates. An isolate should be identified as *L. monocytogenes* if it meets all the other criteria outlined in this chapter.

Table 2 contains the information relating to serological characterization of *Listeria* species. Most of the *L. monocytogenes* isolates from patients and the environment are type 1 or 4. More than 90% of *L. monocytogenes* isolates can be serotyped by using commercial (Difco) sera. All the nonpathogenic species, except *L. welshimeri*, share one or more somatic antigens with *L. monocytogenes*. Serotyping alone without thorough characterization, therefore, is not adequate for identification of *L. monocytogenes*.

TABLE 1.—DIFFERENTIATION OF LISTERIA SPECIES

Species	Hemolytic (Beta)	Nitrate reduction	Utilization			Sheep blood stab	Virulence (mouse test)
			Mannitol	Rhamnose	Xylose		
L. monocytogenes	+	−	−	+	−	+	+
L. invanovii	+	−	−	−	+	+	+
L. innocua	−	−	−	V[a]	−	−	−
L. welshimeri	−	−	−	V[a]	+	−	−
L. seeligeri	+	−	−	−	+	+	−
L. grayi	−	−	+	−	−	−	−
L. murrayi	−	+	+	V[a]	−	−	−

[a] V, variable.

TABLE 2.—SEROLOGY OF LISTERIA SPECIES

Species	Serotype
L. monocytogenes	1/2A, 1/2B, 1/2C, 3A, 3B, 3C, 4A, 4AB, 4B, 4C, 4D, 4E, "7".
L. invanovii	5.
L. innocua	4AB, 6A, 6B, Un[a].
L. welshimeri	6A, 6B.
L. seeligeri	1/2B, 4C, 4D, 6B, Un[a].

[a] Un, undefined.

Of the biochemical tests, the rhamnose and xylose fermentation patterns are essential for differentiating *Listeria* species. Exceptions are *L. monocytogenes* and *L. innocua*, both of which do not ferment xylose. These two species can be separated, however, by the absence of hemolytic activity in *L. innocua*, as demonstrated by sheep blood agar stab or the CAMP test, described below. *L. seeligeri*, on the other hand, may be slightly hemolytic on sheep blood agar. Because it shares antigens with *L. monocytogenes*, it might be incorrectly identified without the xylose-rhamnose reactions and/or mouse pathogenicity. The importance of doing a complete characterization of each isolate cannot be overemphasized. Partial characterization, although accurately done, may be misleading.

The Christie-*A*tkins-*M*unch-*P*eterson (CAMP) test (Table 3 and Figure 1) is useful in confirming species. The test is performed by streaking a *beta*-hemolytic *Staphylococcus aureus* and a *Rhodococcus equi* culture in parallel and diametrially opposite to each other on a sheep blood agar plate, with several test cultures streaked parallel to one another, but at right angles to and between the *S. aureus* and *R. equi*. After incubation at 35° C for 24–48 h, the plates are examined for hemolysis. *L. monocytogenes* hemolysis is enhanced in the zone of influence of the *S. aureus* streak; *L. ivanovii* hemolysis is enhanced near the *R. equi* streak, and *L. seeligeri* is hemolytic near the *S. aureus* streak. The other species remain nonhemolytic. Hemolysis is more easily read when the blood agar is thinner than usual. The *L. monocytogenes* reaction is often optimal at 24 h rather than 48 h. In order to obtain enough *R. equi* to give a good streak of growth, the inoculum slant culture usually requires more than 24-h incubation. Use of known control *Listeria* species on a separate sheep blood agar plate is recommended. Sheep blood agar plates should be as fresh as possible.

TABLE 3.—CAMP TEST REACTIONS OF *Listeria* SPECIES

Species	*Hemolytic reaction*	
	Staphylococcus aureus	Rhodococcus equi
L. monocytogenes	x+	x−
L. ivanovii	x−	x+
L. innocua	x−	x−
L. welshimeri	x−	x−
L. seeligeri	x+	x−

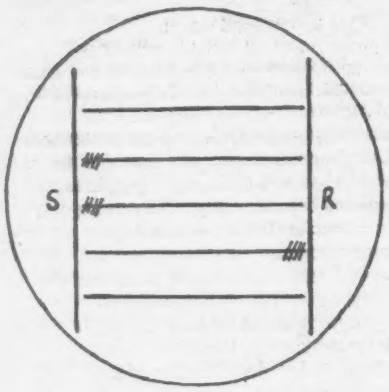

Figure 1. CAMP test for *Listeria monocytogenes:* Inoculation pattern of the sheep blood agar plate. Horizontal lines represent streak inoculations of 5 test strains. Vertical lines represent streak inoculations of *Staphylococcus aureus* (S) and *Rhodococcus equi* (R). Hatched lines indicate (diagrammatically only) the locations of hemolysis enhancement regions.

Refrigeration at 4 °C is recommended for handling, storing, and shipping materials to be analyzed for *L. monocytogenes,* which will grow, although slowly, at this temperature. However, if the test laboratory sample is already frozen, it should be kept frozen until it is ready for analysis.

A. Equipment and Materials

1. Balance (gram range)
2. Cover slips, glass
3. Erlenmeyer flask, 500 ml
4. Fermentation tubes (Durham)
5. Grease pencil or magic marker
6. Incubators, 30 and 35 °C
7. Immersion oil
8. Inoculating loops
9. Inoculating needle
10. Microscope slides
11. Needle, 23 gauge, ⅝ inch
12. Phase microscope with oil immersion objective
13. Petri plates
14. Pipets, 25, 10, and 1 ml
15. Tubes, 16 x 125 mm, screw-cap
16. Blender and jars or stomacher and stomacher bags
17. Syringe, tuberculin, sterile, disposable
18. Dissecting regular or low power microscope with illuminator

B. Media and Reagents (*See* Formulations at end of This Chapter)

(*Note:* Alternative Companies may be Used When the Products Are Equivalent.)

1. Acetic acid, 5 N
2. Acriflavin HCl (Sigma)
3. Agar (Difco Bacto)
4. *alpha*-Naphthylamine
5. *alpha*-Naphthol, 5% in absolute ethanol
6. Blood agar base No. 2 (Oxoid)
7. Cycloheximide (Sigma)
8. Sheep blood, defibrinated
9. Ethanol, absolute
10. Fluorescent antibody (FA) buffer (Difco)
11. Glycine anhydride (Sigma)
12. Gram stain kit
13. Hydrogen peroxide solution, 3% (R18)
14. KOH, 40% solution (R77)
15. *Listeria*-typing sera set (Difco)
16. Lithium chloride (Sigma)
17. Moxalactam (Sigma)
18. Nalidixic acid (sodium salt) (Sigma)
19. NaCl, physiological saline solution, 0.85% (R76)
20. Nutrient broth (M101)
21. Phenylethanol agar (Difco)
22. Purple carbohydrate fermentation broth base (M116), containing 0.5% solutions of dextrose, esculin, maltose, rhamnose, mannitol, and xylose
23. SIM motility medium (Becton Dickinson Microbiology Systems:BDMS)
24. Sulfanilic acid (crystal)
25. Trypticase soy broth (M137)
26. Trypticase soy agar (M136)
27. Tryptose (Difco Bacto)
28. Triple sugar iron (TSI) agar (M134)
29. Yeast extract (BDMS)

C. Enrichment Procedure

Care should be taken to make the sample representative of the food's outer surface as well as its interior.

Add 25 ml liquid or 25 g cream or solid test material to 225 ml enrichment broth (EB) in blender or stomacher. Blend or stomach as required for thorough mixing. Incubate EB culture in blender jar or stomacher bag, or transfer to 500 ml Erlenmeyer flask. Incubate EB culture for 2 days at 30°C.

D. Isolation Procedure

At 24 h and at 2 days, streak EB culture onto modified McBride agar (MMA) and onto *L*ithium chloride-*P*henylethanol-*M*oxalactam (LPM) agar. Incubate MMA plates at 30°C for 48 h and LPM plates at 30°C for 48 h. Examine MMA plates for suspect colonies by using beamed white light powerful enough to illuminate plate well, striking plate bottom at 45° angle (Figure 2). When examined in this oblique-transmitted light from an eye position directly above the plate (i.e., at 90° to the plate) either directly or via a low power microscope or dissecting microscope (with mirrors detached), colonies of *Listeria* species appear blue-gray to blue on MMA. On LPM plating medium the colonies appear sparkling blue (bluish crushed glass) or white. The use of positive and negative control colonies on MMB and LPM (not the test plates) to attune the observer's eyes is recommended. Although there are variations in the components of the optical systems used, the important points are the 45° angle of incident light and the 90° angle of emergent light. Pick 5 or more typical colonies to trypticase soy agar with 0.6% yeast extract (TSA-YE), streaking for isolated colonies. Incubate TSA-YE plates at 30°C for 24 h or until growth is satisfactory.

E. Identification Procedure

Isolates must be identified by the following classical tests (E1–E13). Rapid kits are available to facilitate biochemical testing to genus level (see E14). In addition, there are now rapid novel methods available (see section]) employing monoclonal antibodies and gene probes which provide advance indication of the presence of *Listeria* species (genus only) in 48-h samples of the enrichment broth or of *L. Monocytogenes* colonies on the isolation agar (these tests are optional).

1. Examine TSA-YE plates for typical colonies. Using the oblique illumination system (Henry illumination) already described, the colonies will have a similar appearance to that described for MMB (section D). Again, the use of known controls on TSA-YE is recommended.

2. Pick a typical colony and examine by wet mount, using 0.85% saline for suspending medium and oil immersion objective of phase-contrast microscope. Be sure to choose a colony with enough growth to make a fairly heavy suspension; emulsify thoroughly. If too little growth is used, the few cells

present will stick to the glass slide and appear nonmotile. *Listeria* species appear as slim, short rods with slight rotating or tumbling motility. Always compare with known culture. Cocci, large rods, or rods with rapid, swimming motility are not *Listeria* species.

3. Test typical colony for catalase. *Listeria* species are catalase-positive.

4. Gram stain 16- to 24-h cultures. All *Listeria* species are small, Gram-positive rods, but Gram stain reaction can be variable with older cultures.

5. Pick a typical colony to a tube of trypticase soy broth supplemented with 0.6% yeast extract (TSB-YE) for inoculating biochemicals and fermentations. Incubate at 35°C for 24 h. This culture may be kept at 4°C for several days and used as inoculum.

6. Inoculate heavily (from a TSA-YE colony) sheep blood agar by stabbing plates that have been poured thick and dried well (check for moisture before using). Draw grid of 20–25 spaces on plate bottom. Stab one culture per grid space. Always stab positive controls (*L. ivanovii* and *L. monocytogenes*) and a negative control (*L. innocua*). Incubate for 48 h at 35°C.

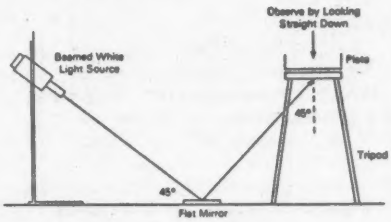

Figure 2. Examination of plates for suspect colonies of *Listeria* species. Light source used, Bausch & Lomb Nicholas Illuminator, Cat. No. 31–33–05–28; *see* a current Fisher Catalog. Ref. Seeliger, H.P.R., 1961, Listeriosis, Hafner Publishing Co., New York, NY. Note: some observers prefer a concave mirror.

7. Examine blood agar plates containing culture stabs by using a bright light. *L. monocytogenes* and *L. seeligeri* produce a slightly cleared zone around stab. *L. innocua* shows no zone of hemolysis, whereas *L. ivanovii* produces as well-defined zone of clearing around stab. Do not try to differentiate species at this point, but note nature of the hemolytic reaction.

8. Using the TSB–YE culture, inoculate urea agar slant by covering the slant well without stabbing the butt. Incubate at 35 °C for 5 days. Observe daily for development of a purple color (a positive test). *Listeria* species do not hydrolyze urea and no color should develop.

9. Nitrate reduction test. To perform this test, use a TSB–YE culture to inoculate nitrate broth. Incubate at 35 °C for 5 days. Add 0.2 ml reagent A, followed by 0.2 ml reagent B (*see* Media No. 6 at end of this chapter). A red color indicates the presence of nitrite, i.e., nitrate has been reduced. If no color develops, add powdered zinc and let stand 1 h. A developing red color indicates that nitrate is still present and has not been reduced. Only *L. murrayi* reduces nitrates.

As an alternative procedure, add 0.2 ml reagent A followed by 0.2 ml reagent C. An orange color indicates reduction of nitrate. If not color develops, add 0.2 ml cadmium reagent. The development of an orange color indicates unreduced nitrate. This procedure eliminates the use of the carcinogen *alpha*-naphthylamine.

10. Inoculate SIM motility medium from TSB–YE. Incubate for 7 days at room temperature. Observe daily. *Listeria* species are motile, giving a typical umbrella-like growth pattern.

11. Inoculate an MR–VP medium tube, using the TSB–YE culture. Incubate at 35 °C for 48 h. Remove 1 ml to a clean tube and add 0.6 ml *alpha*-naphthol solution followed by 0.2 ml 40% KOH solution. Shake and examine. A strong red color indicates a positive test. Incubate remaining culture for additional 2 days. Add methyl red to tube; it should turn red for a positive test. *Listeria* species are both MR- and VP-positive.

12. Using the TSB–YE culture, inoculate TSI by streaking slant and stabbing butt. Incubate up to 5 days at 35 °C *Listeria* species give an acid slant and an acid butt, but no H_2S.

13. From TSB–YE culture, inoculate the following carbohydrates set up as 0.5% solutions in purple carbohydrate broth: dextrose, esculin, maltose, rhamnose, mannitol, and xylose. Incubate 7 days at 35 °C. *Listeria* species produce acid with no gas or no reaction. Consult Table 1 for xylose-rhamnose reactions of *Listeria* species. All species should be positive for dextrose, esculin, and maltose. All *Listeria* species except *L. grayi* and *L. murrayi* should be mannitol-negative.

14. Purified isolates with known hemolytic reactions can be rapidly identified (to *genus* level only) using the commercial kits 20 S™ (Analytical Products, Plainview, N.Y. USA) and Gram Positive Identification card (Vitek Systems, McDonnell Douglas, Hazelwood, MO). The later kit is used in conjunction with the Vitek Automicrobic System.

F. Serology

This test is used when epidemiological considerations are crucial. Use TSB–YE culture to inoculate tryptose broth. Make two successive transfers of cultures incubated in tryptose broth for 24 h. at 35 °C. Make a final transfer to two tryptose agar slants and incubate 24 h. at 35 °C. Wash both slants in a total of 3 ml Difco FA buffer and transfer to sterile 16 x 125mm screw-cap tube. Heat in water bath at 80 °C for 1 h. Spin at 1600 x T3g for 30 min. Remove 2.2–2.3 ml of supernatant fluid and resuspend pellet in remainder of buffer. Follow manufacturer's recommendations for sera dilution and agglutination procedure. If flagellar (H) and sub-factor (0) serotyping is required, the procedures can be obtained from the Division of Microbiology, FMMD Branch, FDA, 200 C Street, SW., Washington, DC 20204.

G. Mouse Pathogenicity (Optional)

Grow isolate for 24 h at 35°C in TSB-YE. Transfer to two tubes of TSB-YE for another 24 h at 35°C. Place a total of 10 ml culture broth from both tubes into 16 x 125 mm tube and spin at 1600 x *g* for 30 min. Discard supernatant and resuspend pellet in 1 ml 0.85% saline diluent. This suspension will contain approximately 10^{10} bacteria/ml and the actual concentration should be determined by a pour or spread plate count. Inject (i.p.) 16- to 18-g Swiss white mice (5 mice/culture) with 0.1 ml of the concentrated suspension. Each mouse will receive 10^9 bacterial cells. Observe for death over a 7-day period. Nonpathogenic strains will not kill, but 10^9 pathogenic cells will kill, usually within 5 days. This test should be controlled with known pathogenic and nonpathogenic strains.

H. CAMP Test. CAMP Test Cultures of *S. Aureus* and *R. Equi* Are Available From the Division Microbiology, FMMD Branch, FDA, 200 C Street, S.W, Washington, DC 20204.

Streak *beta*-hemolytic *S. aureus* (CIP 5710 or NCTC 7428) and *R. equi* (NCTC 1621) vertically on sheep blood agar. Separate vertical streaks so that test strains may be streaked horizontally between them without quite touching them. After 24 and 48 h incubation at 35°C, examine plates for hemolysis in the zone of influence of the vertical streaks. Figure 1 shows the arrangement of the streak cultures on a CAMP plate. *L. monocytogenes* hemolysis is enhanced in the vicinity of the *S. aureus* streak; *L. invanovii* hemolysis is enhanced in the vicinity of the *R. equi* streak; and hemollysis of *L. seeligeri* is

enhanced near the *S. aureus* streak. The other species remain nonhemolytic in this test. The CAMP test differentiates *L. ivanovii* from *L. seeligeri* and can differentiate a weakly hemolytic *L. seeligeri* (that may have been read as nonhemolytic) from *L. welshimeri*. Isolates giving reactions typical for *L. monocytogenes* except for the hemolysin production should be CAMP-tested before they are identified as nonhemolytic *L. innocua*. (A factor easily prepared from *S. aureus* cultures can be used to enhance hemolysis by *L. monocytogenes* and *L. seeligeri* in sheep blood agar plates.)

I. Interpretation of Analyses Data for Speciation

All *Listeria* spp. are small Gram-positive rods that demonstrate motility in wet mount and in SIM. They are catalase-positive, do not hydrolyze urea, and produce acid slant and acid butt in TSI without production of H_2S. They utilize dextrose, esculin, and maltose, and some species utilize mannitol, rhamnose, and xylose with production of acid. All species give +/+ reactions in MR-VP broth. An isolate utilizing mannitol with acid production is *L. grayi* *L.* or *murrayi*. Nitrate reduction differentiates between the two because only *L. murrayi* reduces nitrate.

L. monocytogenes. *L. ivanovii*, and *L. seeligeri* produce hemolysis in sheep blood stabs and are consequently CAMP test-postive. Of the three, only *L. monocytogenes* fails to utilize xylose and is positive for rhamnose utilization. The difficulty in differentiating *L. ivanovii* from *L. seeligeri* can be resolved by the CAMP test. *L. seeligeri* shows enhanced hemolysis at the *S. aureus* streak. *L. ivanovii* shows enhanced hemolysis at the *R. equi* streak. Of the nonhemolytic species, *L. innocua* may give the same rhamnose-xylose reactions as *L. monocytogenes* but is negative in the CAMP test. *L. innocua* is the only species that sometimes gives negative results for utilization of both rhamnose and xylose. *L. welshimeri* that is rhamnose-negative may be confused with a weakly hemolytic *L. seeligeri* unless resolved by the CAMP test.

After all other results are available, the serotyping of *Listeria* isolates becomes meaningful. Biochemical, serological, and pathogenicity data are summarized in Tables 1–3. All data collection must be completed before speciation.

J. Rapid Presumptive Identification Methods (Optional)

Several rapid methods are available for identifying *Listeria* isolates to genus or species (*L. monocytogenes*) level. These methods can accelerate the identification process. The methods are supplementary to the conventional procedures and are not intended as substitutes for them since their reliability and acceptability has not yet been fully evaluated by widespread use. Details of the methods can be found in the provided references.

1. Identification in enrichment culture. Members of the genus *Listeria* can be identified in the 48-h enrichment broth culture by using a commercial ELISA kit (Organon Teknika Corp., Durham, NC) (9) or a radiolabeled DNA probe hybridization kit (GeneTrak, Framingham, MA) (10). The genus *Listeria* can be detected directly in enrichment broths containing esculin and iron (11).

(2) Identification of colonies on isolation agar. Colonies of *L. monocytogenes* on LPM agar can be identified by using an FDA-developed radiolabeled gene probe that is specific for a DNA sequence located either within or close to a hemolysin gene (12). Colonies of *Listeria* spp. can be recognized on an esculin and iron selective medium (Oxford formulation) which is marketed by Oxoid Ltd., Basingstoke, Hampshire, U.K.

K. Quantification (optional)

There are no official methods of quantification. However, if a sample is directly implicated as being a cause of foodborne listeriosis, an attempt should be made to quantitate *Listeria* prior to enrichment by direct plate count (on both MMB and LPM) and/or by MPN procedure using EB. Such data would be useful in estimating infectious-dose values.

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Chapter 29. Media Supplement

1. Sheep Blood Agar

Blood agar base (Oxoid No. 2)	93 ml
Sheep blood, defibrinated	5 ml

Rehydrate and sterilize as recommended by manufacturer. Agar and blood should both be at 45–46°C before adding blood and pouring plates. Sheep blood agar plates are available commercially.

2. Purple Carbohydrate Fermentation Broth Base (Ml 16)

Purple broth base (BBL)	15 g
Distilled water	900 ml

Add purple broth base to distilled water. Dispense 9 ml to 16×125 mm tubes containing a Durham tube.

Autoclave at 121°C for 15 min. Prepare all carbohydrates, except esculin, as sterile 5% solutions. Filter-sterilize. Add 1 ml carbohydrate solution to 9 ml broth base to yield 0.5% carbohydrate in broth.

Add esculin directly into base broth to make a 0.5% solution and autoclave 15 min at 115°C. A 5% solution of esculin at room temperature is a gel that cannot be pipetted.

3. Enrichment Broth (EB), pH 7.3

TSB–YE (*see* No. 11, below) supplemented with:

Acriflavin HC1 (Sigma)	15 mg/L
Nalidixic acid (sodium salt) (Sigma)	40 mg/L
Cycloheximide (Sigma)	50 mg/L

Add the three supplementary ingredients aseptically to TSB–YE after autoclaving and just before use. Make acriflavin and naladixic supplements as 0.5% stock solutions in distilled water. Make cycloheximide supplement as 1.0% stock solution in a 40% solution of ethanol in water. Filter-sterilize all three supplementary ingredients. Add 0.68 ml acriflavin stock solution, 1.8 ml nalidixic stock solution, and 1.15 ml cycloheximide stock solution to 225 ml TSB–YE to achieve the stated amounts in mg/L. EB is commercially available. (*Note*: Cycloheximide is very toxic. Care should be exercised in its disposal, especially if large volumes of EB (and MMA) are used. Refer to a current edition of the Merck Index (Merck Co. Inc., Rahway NJ) for information on its stability properties in relation to disposal methods.)

4. Modified McBride Agar (MMA), pH7.3

Phenylethanol agar (Difco)	35.5 g
Glycine anhydride (Sigma)	10.0 g
Lithium chloride (Sigma)	0.5 g
Cycloheximide (add *after* autoclaving)	200.0 mg
Distilled water	1 liter

Heat to boiling to dissolve agar and dispense into bottles. Autoclave at 121°C for 15 min. Store in refrigerator. Melt and temper agar, and then add cycloheximide (filter-sterilized) before pouring plates. These plates may be stored in refrigerator for 1 week. Do not dry plates. Commercial dehydrated MMA with glycine anhydride is not available.

5. MR–VP Medium (M92)

MR–VP reagents

Methyl red. 0.1 g methyl red in 300 ml 95% ethanol made up to 500 ml in distilled water.

alpha-Naphthol solution. 5% *alpha*-Naphthol in absolute ethanol.

KOH solution. 40% W/V KOH in distilled water.

6. Nitrate Reduction Medium—Nutrient Broth (M101) with 1.0 g/L Potassium Nitrate (M96)

Reagent A. Dissolve 0.5 g *alpha*-naphthylamine in 100 ml 5 N acetic acid by gently heating. Prepare 5N acetic acid by adding distilled water to 28.7 ml of glacial acetic acid (17.4 N) to give a final volume of 100 ml.

Reagent B. Dissolve 0.8 g sulfanilic acid in 100 ml 5*N* acetic acid by gently heating.

Reagent C. Dissolve 1 g *alpha*-naphthol in 200 ml acetic acid.

Zinc powder. For nitrate reduction test, follow directions described in this chapter under E–9.

Cadmium reagent. Place zinc rods in 20% solution of cadmium sulfate for several hours. Draw off precipitated cadmium and add to 1 N HCl.

7. SIM Motility Medium (BBL)

Rehydrate and sterilize according to manufacturer's instructions. Add 6 ml medium per 16 x 125 mm screw-cap tube.

8. Triple Sugar Iron (TSI) Agar (M134)

Use either BBL or Difco TSI, prepared per manufacturer's instructions. Streak and stab butt.

9. Tryptose Broth and Agar for Serology

Difco Bacto tryptose	20 g
NaCl, 0.85% solution	8.5 g
Dextrose	1 g
Agar	15 g
Distilled water	1 liter

For broth, omit agar from formulation.

10. Trypticase Soy Agar with 0.6% Yeast Extract (TSA–YE)

Trypticase soy agar (BBL)	40 g
Yeast extract (BBL)	6 g
Distilled water	1 liter

11. Trypticase Soy Broth with 0.6% Yeast Extract (TSB–YE)

Trypticase soy broth (BBL)	30 g
Yeast extract (BBL)	6 g
Distilled water	1 liter

12. Urea Hydrolysis Medium

Agar	1.5 g
Distilled water	90 ml
Urea agar concentrate (Difco)	1 tube (10 ml)

Add agar to distilled water and heat to boiling to dissolve. Autoclave at 121°C for 15 min. Temper agar to 50–55°C and aseptically add contents of one 10-ml tube of urea agar concentrate. Mix thoroughly. Dispense to sterile tubes and slant generously.

13. Lithium Chloride-Phenylethanol-Moxalactam (LPM) Medium

Phenylethanol agar (Difco)	35.5 g
Glycine anhydride (*Note*: not glycine)	10 g
Lithium chloride	5 g
Moxalactam stock solution, 1% in pH 6.0 phosphate buffer	2 ml
Distilled water	1 liter

Sterilize the medium (without moxalactam) at 121°C for 15 min. Cool to 48–50°C and add filter-sterilized moxalactam solution.

Moxalactam stock solution consists of 1 g of moxalactam salt (ammonium or sodium) in 100 ml of 0.1 M potassium phosphate buffer pH 6.0. Store the filter-sterilized stock solution frozen in 2-ml aliquots.

Moxalactam (E. Lilly Co.) is retailed by Sigma Chemical Co. The LPM medium is most effective in the Henry illumination system when poured thin, i.e., 12–15 ml per standard petri dish. The greater tendency of this agar to dry is avoided by refrigeration and/or rapid use of the plates. LPM basal medium is commerically available as a powder.

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